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Replication of *Orgyia pseudotsugata* Baculovirus DNA: *lef-2* and *ie-1* Are Essential and *ie-2*, *p*34, and Op-*iap* Are Stimulatory Genes

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A transient DNA replication assay was used to identify genes located within m.u. 90.5–7.0 of the *Orgyia pseudotsugata* multinucleocapsid nuclear polyhedrosis virus (OpMNPV) genome that influenced replication of a reporter plasmid containing an OpMNPV origin of replication, when cotransfected into uninfected *Lymantria dispar* cells. The viral transactivator *ie-1* and a 2.4-kb subclone were found to be essential for replication. The 2.4-kb region was sequenced and open reading frames were identified. Replication assays using subclones from this region identified a gene called late expression factor 2 (*lef-2*), as the essential replication gene. The OpMNPV *lef-2* gene encodes a protein with a predicted molecular weight of 22.7 kDa (204 amino acids) and exhibits 54.7% amino acid sequence identity with its homolog in the genome of the *Autographa californica* MNPV. Transcriptional mapping using both Northern blot and S1 nuclease protection assays demonstrated that OpMNPV *lef-2* was expressed at both early and late times postinfection as a transcript of about 1.6 kb. The early transcript initiated approximately 30 nt downstream of a TAATA box, whereas the late transcript initiated from within a late promoter consensus motif. In addition, we identified three genes stimulatory for DNA replication including two OpMNPV transcriptional activators (*ie-2* and *p*34) and Op-*iap*, which is the functional analog of the AcMNPV *p35* gene that inhibits apoptosis in AcMNPV-infected *Spodoptera frugiperda* cells.

INTRODUCTION

Baculoviruses have large, covalently closed, circular, double-stranded DNA genomes of 88 to over 166 kb depending on the virus strain (Blissard and Rohrmann, 1990). Although they have achieved widespread use as expression vectors, little is known about how they replicate their DNA. Recently, putative origins of DNA replication have been identified in several baculoviruses. In two of these, the *Autographa californica* multinucleocapsid nuclear polyhedrosis virus (AcMNPV) and the *Orgyia pseudotsugata* MNPV (OpMNPV), multiple putative replication origins have been identified that are composed of either unique or repeated DNA (Pearson *et al.*, 1993; Kool *et al.*, 1993; Leisy and Rohrmann, 1993; Ahrens *et al.*, 1995b). Despite the fact that the genomes of these two viruses are of similar size and large portions of the genomes show a colinear pattern of organization of homologous genes (Leisy *et al.*, 1984), these putative replication origins demonstrate limited sequence homology. In addition, plasmids containing a putative origin from one of these viruses are replicated only very poorly in cells infected with the heterologous virus (Pearson *et al.*, 1993; Ahrens *et al.*, 1995b). This suggests that, although the viruses have genomes of similar structure and organization, the DNA replication apparatus of these two viruses is highly specific.

In order to characterize the factors which govern the specificity of the baculovirus replication apparatus, we have employed transient replication assay protocols to identify genes required for baculovirus DNA replication. Using this strategy, a set of six essential and three stimulatory genes were identified for AcMNPV (Kool *et al.*, 1994). In addition, a limited subset of OpMNPV cosmid and plasmid clones that support replication have been described and we have begun to identify and characterize the essential replication genes in the OpMNPV genome. We previously reported that the OpMNPV *lef-1* (Ahrens and Rohrmann, 1995) and *lef-3* genes (Ahrens *et al.*, 1995a) are essential for replication of an OpMNPV origin-containing plasmid in uninfected *Lymantria dispar* cells. We have also shown that OpMNPV homologs of DNA polymerase and helicase genes are also essential in this replication assay (Pearson *et al.*, 1993; Ahrens and Rohrmann, 1995).

In this report, we describe studies using the transient replication assay to characterize subclones from m.u. 90.5–7.0 of the OpMNPV genome. We determined that two genes (*ie-1* and *lef-2*) are essential, whereas two others (*ie-2* and *p*34) are stimulatory for DNA replication. In addition, we show that another gene, OpMNPV *i*nhibitor of *a*poptosis (Op-*iap*), which can substitute for AcMNPV *p35* in blocking apoptosis in *Spodoptera frugiperda* cells, stimulated OpMNPV DNA replication.

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MATERIALS AND METHODS

Cells and cosmids

The *L. dispar* (IPLB-Ld-652Y) cell line was propagated at 27° in TNM-FH medium supplemented with 10% (v/v) fetal bovine serum, penicillin G (50 units/ml), streptomycin (50 μg/ml, Whitaker Bioproducts), and fungizone (Amphotericin B, 375 ng/ml, Flow Laboratories), according to standard procedures (Summers and Smith, 1987). The OpMNPV cosmids are described in Leisy *et al.* (1984).

Replication assay and quantification of replication efficiency

The replication assay was described previously (Ahrens and Rohrmann, 1995). The replication efficiency of the origin-containing reporter plasmid pHdN cotransfected with the essential replication genes alone or with additional stimulatory genes was quantified with a PSI-486 Phosphorimager SI & Imagequant Workstation (Molecular Dynamics), using the Scanner Control SI-PDSI version 1.0 and Imagequant 4.1 software packages. The relative values of stimulation of DNA replication represent an average value of levels measured for two or more independent replication assays (if not indicated otherwise).

Recombinant DNA

Cloning protocols involved the use of pBlueScribe (pBS^-) or pBlueScript (pKS^-) vectors (Stratagene, Inc.), the former modified by the addition of a *Bgl*II site in the polylinker (Gombart *et al.*, 1989). Clones were transformed into *Escherichia coli* JM83 or DH5α strains according to standard procedures (Sambrook *et al.*, 1989). Previously described clones include the following: the reporter plasmid pHdN (m.u. 8.1–11.1) and pDNApol (m.u. 43.9–47.0, containing the OpMNPV DNA polymerase gene) (Ahrens and Rohrmann, 1995), pOp5 (Ahrens *et al.*, 1995b), phel (m.u. 62.8–66.9 containing the helicase gene) (Ahrens *et al.*, 1995a), and pAcJI (4.7 kb, m.u. 3.2–6.9), an *Xho*I fragment into *Xho*I cut pACYC (Rohrmann *et al.*, 1982). Subclones from the *Hin*dIII-A fragment are shown in Fig. 1b and were cloned as follows: pCA25 (11.2 kb, m.u. 90.5–99.0) and pCA24 (9.25 kb, m.u. 0–7.0), *Hin*dIII/*Eco*RI fragments into *Hin*dIII–*Eco*RI cut pBS^-; pGR4 (2.4 kb, m.u. 5.2–7.0), an *Xba*I/*Hin*dIII fragment into *Xba*I–*Hin*dIII cut pUC19; pCA11 (9.3 kb, m.u. 89.7–96.7), a *Cla*I fragment from cosmid 47 (Leisy *et al.*, 1984) into *Acc*I cut pBS^-. POp47*Sal*-IE-1 (called pIE-1 in this paper) (Theilmann and Stewart, 1994), pIE2-E2.3 (called pIE-2 in this paper) (Theilmann and Stewart, 1992a), and p34*Sma*I (Wu *et al.*, 1993a) were the gift of D. Theilmann. pOp47B/E (5.0 kb, m.u. 0–3.8), an *Eco*RI/*Bam*HI fragment cloned into *Eco*RI–*Bam*HI cut pBS was also provided by D. Theilmann. Several subclones of pGR4 were generated for the identification of ORFs essential for DNA replication from this 2.4-kb region and are shown in Fig. 3a: pCA30 (1.3 kb, m.u. 5.65–6.65), a *Sal*I fragment into *Sal*I cut pBS^-; pCA35 (1.2 kb, m.u. 5.6–6.5), a *Pvu*II fragment into *Sma*I cut pBS^-; and pCA35*Sph*IΔ, (an internal 150 bp *Sph*I deletion from pCA35).

For the studies on genes that transactivate OpMNPV DNA replication, the following clones were used in addition to pIE-2 and p34*Sma*I: *Hin*dIII K (4.0 kb, m.u. 22.4–25.4), a *Hin*dIII fragment cloned into *Hin*dIII cut pBS^-; pCA16 (1.5 kb, m.u. 22.4–23.6) and pCA18 (1.5 kb, m.u. 24.2–25.4), *Hin*dIII/*Sst*I fragments cloned into *Hin*dIII–*Sst*I cut pBS; pCA17 (1.0 kb, m.u. 23.6–24.2), an *Sst*I fragment cloned into *Sst*I cut pBS; pOp-*iap* (1.4 kb, m.u. 24.4–25.45) was a gift from Dr. Lois Miller and is described in Birnbaum *et al.* (1994).

POp47Pst*6.0* B105 and pOp47Pst*6.0* B23 are deletion clones of pOp47Pst*6.0*, which contains *ie-1* and open reading frame (ORF) 146 (Theilmann and Stewart, 1994), and were the gift of Dr. D. Theilmann. ORF 146 contains 196 amino acids. Both deletion clones contain the entire *ie-1* gene but retain only the amino-terminal 107 (pOp47Pst*6.0* B105) or 21 (pOp47Pst*6.0* B23) amino acids of ORF 146.

DNA sequencing and analysis

Exonuclease III deletion clones (Henikoff, 1987) for DNA sequence determination and replication analyses were generated from pGR4 in both directions, and the nucleotide sequence of this 2.4-kb region was determined using methods described previously (Ahrens *et al.*, 1995a). The nucleotide sequence and the predicted protein sequences were analyzed with the GCG suite of sequence analysis programs (Devereux *et al.*, 1984), Version 7.2-UNIX (1992).

RNA isolation, transcriptional analysis by Northern hybridization, and S1 nuclease mapping

Both the isolation of total RNA from a time course of OpMNPV-infected *L. dispar* cells based on a modified version (Gross and Rohrmann, 1993) of the original protocol by Glisin *et al.* (1974), as well as the transcriptional mapping of RNA's of selected time points with S1 nuclease (Favaloro *et al.*, 1980) have been described previously (Ahrens and Rohrmann, 1995). S1 nuclease protected fragments for the 5′ and 3′ mapping of *lef-2* transcripts were analyzed on 5% polyacrylamide, 4.6 *M* urea gels and visualized by autoradiography. The sequence ladder used for sizing the protected fragments was generated with the Sequenase Version 1.0 T7 DNA sequencing kit using bacteriophage M13mp18 DNA and the M13 (−40) sequencing primer (USB).

The clone used to generate a strand-specific probe for the analysis of the temporal expression of *lef-2* was derived by cutting pCA30 with *Sph*I and religating the remaining vector-containing 3.9-kb fragment to produce

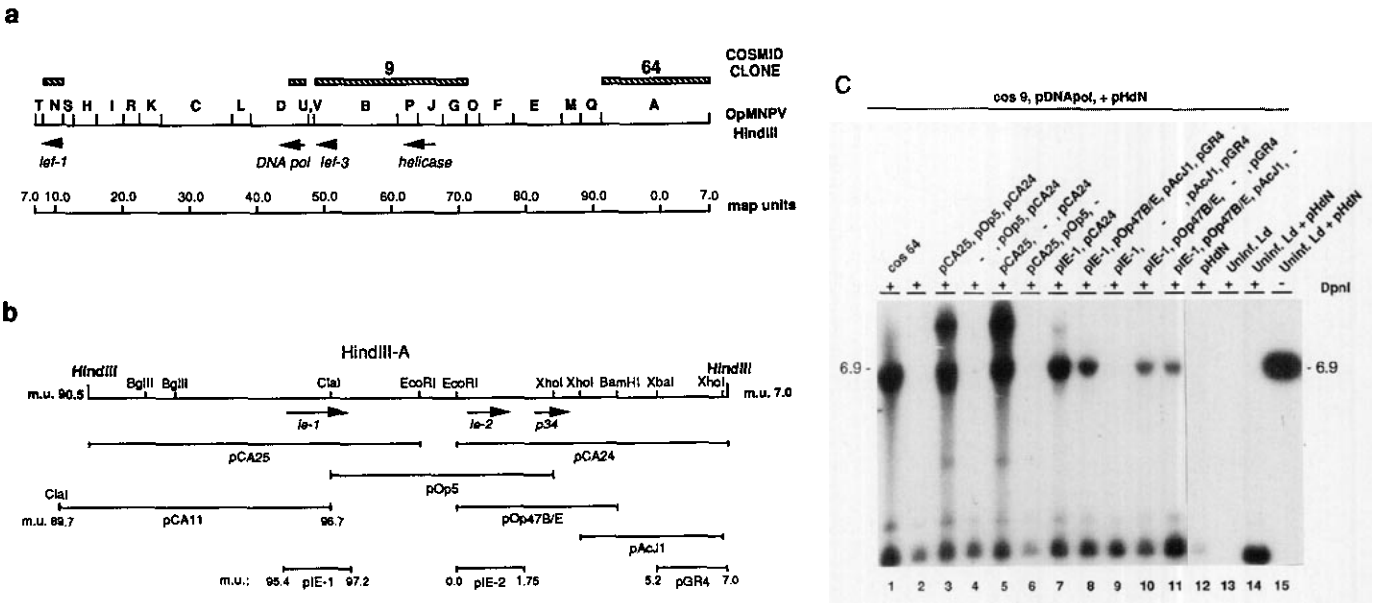

FIG. 1. Identification of sequences essential for OpMNPV DNA replication contained on the *Hin*dIII-A fragment (cosmid 64). (a) *Hin*dIII restriction map of the OpMNPV genome with a schematic representation of the previously identified subset of cosmids and plasmids (cosmid 9, 64, pDNApol, and pHdN; crosshatched bars), which supply all the virus-encoded *trans*-acting factors essential for replication of the origin-containing reporter plasmid pHdN (m.u. 8.1–11.1) (Ahrens and Rohrmann, 1995). The locations of previously identified essential OpMNPV DNA replication genes (*lef-1, DNA polymerase, lef-3, and helicase*) are shown and map units are indicated below the map. (b) Map of the *Hin*dIII-A fragment (m.u. 90.5–7.0) with selected restriction endonuclease sites and a schematic representation of the subclones used to identify sequences essential for DNA replication within *Hin*dIII-A. Map units are shown for two subclones that contain essential replication genes, pIE-1 and pGR4, for a plasmid containing a gene stimulatory for DNA replication (pIE-2) and for pCA11. (c) Replication analysis of subclones of *Hin*dIII-A for their ability to supply essential replication gene(s). The replication signals result from transfection mixtures containing cosmid 9, pDNApol, pHdN, and the following additional clones: Lane 1, cosmid 64; lane 2, no additional clones; lane 3, pCA25, pOp5, and pCA24; lane 4, pOp5 and pCA24; lane 5, pCA25 and pCA24; lane 6, pCA25 and pOp5; lane 7, pIE-1 and pCA24; lane 8, pIE-1, pOp47B/E, pAcJ1, and pGR4; lane 9, pIE-1, pAcJ1, and pGR4; lane 10, pIE-1, *pOp47B/E, and pGR4; lane 11, pIE-1, pOp47B/E, and pAcJ1. Lane 12–15 show controls routinely included in the replication assays described in* this report and contain the following: lane 12, uninfected *L. dispar* cells transfected with pHdN alone (shows no replication of pHdN); lane 13, DNA from uninfected *L. dispar* cells (shows no hybridization with the probe); lanes 14 and 15, plasmid DNA mixed with uninfected cell DNA and digested with or without *Dpn*I, respectively (shows that digestion is complete under the conditions used in our experiments). The number to the left of the blot corresponds to the size (in kb) of the hybridized band of linearized pHdN.

pCA39. Labeled cRNA transcripts were generated using T3 RNA polymerase (Promega) and [^{32}P]UTP from pCA39 linearized with *Sst*II and hybridized to Northern blots of a time course of RNA isolated from infected cells as previously described (Ahrens and Rohrmann, 1995).

Nucleotide sequence accession number

The nucleotide sequence data reported in this paper will appear in the GSDB, DDBJ, EMBL, and NCBI nucleotide sequence databases under Accession No. D50053.

RESULTS

Identification of essential replication genes within m.u 90.5–7.0 of the OpMNPV genome

We previously described a limited subset of cosmid and plasmid clones of the OpMNPV genome that suffice to supply all the virus-encoded *trans*-acting genes essential for DNA replication of the origin-containing reporter plasmid pHdN. These include cosmids 9 and 64, pDNApol and pHdN, Fig. 1a, crosshatched bars (Pearson *et al.*, 1993; Ahrens and Rohrmann, 1995). Subsequently, we identified four essential OpMNPV DNA replication genes encoded by the plasmid or cosmid clones, including the DNA polymerase gene (pDNApol) (Pearson *et al.*, 1993), *lef-1* (pHdN) (Ahrens and Rohrmann, 1995), the helicase gene (Ahrens and Rohrmann, 1995), and *lef-3* (cosmid 9) (Ahrens *et al.*, 1995a). Omission of cosmid 64 from a transfection mixture containing cosmid 9, pDNApol, and pHdN (which contains a viral *cis*-acting origin of replication in addition to the essential replication gene *lef-1*), results in the abrogation of the replication signal (Fig. 1c, lane 2 versus lane 1). This indicated that additional essential replication genes were contained on cosmid 64, which covers the region from m.u. 90.5–7.0 of the OpMNPV genome.

In order to identify the essential *trans*-acting factors contained on cosmid 64, we analyzed several large subclones for their ability to supply the essential factors (Fig. 1b). Cosmid 64 could be substituted in a cotransfection mixture containing a limited subset of clones (cosmid 9, pDNApol, pHdN) by three overlapping subclones pCA25, pOp5, and pCA24 (Fig. 1c, lane 3). Eliminating pCA25 or

pCA24 from this set of three subclones resulted in the abrogation of the replication signal (Fig. 1c, lanes 4 and 6), whereas omission of pOp5 had no effect (Fig. 1c, lane 5), indicating that cosmid 64 contains at least two essential replication genes. When pCA25 was replaced by pCA11 (Fig. 1b, m.u. 89.7–96.7), no replication signal was observed (data not shown), suggesting that an essential gene might be located near the *Cla*I site at m.u. 96.7, since it is the only area of *Hin*dIII A that was not covered with a significant overlap by the clones tested (Fig. 1b, pCA11 and pOp5 both terminate at the *Cla*I site). The viral transactivator gene *ie-1* spans this site (Theilmann and Stewart, 1991) and a plasmid containing the *ie-1* gene (Fig. 1b; pIE-1, m.u. 95.4–97.2) could substitute for pCA25 (Fig. 1c, lane 7). In addition to *ie-1*, pIE-1 contains all but the last 16 carboxyl-terminal amino acids of an ORF (Theilmann and Stewart, 1991), which shares 50% amino acid identity to its homolog from AcMNPV (ORF 146) (Ayres *et al.*, 1994) and that we have accordingly termed OpMNPV ORF 146 (196 amino acids). Two deletion clones, pOp47*Pst*6.0 B105 and pOp47*Pst*6.0 B23 (see Material and Methods), which retain *ie-1* expression but contain 107 and 21 amino acids of ORF 146, respectively, were able to substitute for pIE-1 in supplying the essential replication gene (data not shown). Therefore, *ie-1* is the essential gene for OpMNPV DNA replication contained on pIE-1.

To further localize the essential replication gene(s) on pCA24, three overlapping subclones, pOp47B/E, pAcJI, and pGR4 (Fig. 1b) were tested for their ability to substitute for pCA24 in the transient complementation assay. In combination, these subclones could substitute for pCA24 when cotransfected along with pIE-1, pDNApol, pHdN, and cosmid 9 (Fig. 1c, lane 8). When each of these three subclones was omitted individually from an identical transfection mixture, we found that omission of pAcJI or pGR4 still resulted in replication (Fig. 1c, lanes 10 and 11), while omission of pOP47B/E almost completely abrogated the replication signal (Fig. 1c, lane 9). However, the replication signal was still visible on longer exposures (data not shown), indicating that pOp47B/E likely contains one or more genes that highly stimulated OpMNPV DNA replication. When both pAcJI and pGR4 were left out together from a transfection mixture containing cosmid 9, pDNApol, pHdN, and pOp47B/E, no replication was observed (see below). This suggested that another essential replication gene was contained within the region covered by both pAcJI and pGR4 (Fig. 1b, m.u. 5.2–6.9).

Controls for this and following experiments are shown in Fig. 1c, lanes 12–15, and indicate the following: uninfected *L. dispar* cells transfected with pHdN alone show no replication of pHdN (lane 12); DNA from uninfected *L. dispar* cells does not hybridize with the probe (lane 13); plasmid DNA mixed with uninfected cell DNA and digested with or without *Dpn*I shows that digestion is complete under the conditions used in our experiments (lanes 14 and 15).

In order to identify the essential replication gene(s) contained within pGR4, its nucleotide sequence was determined.

Nucleotide sequence of the 5.7–7.0 m.u. region of the OpMNPV genome

The 2.4-kb *Hin*dIII–*Xba*I insert of pGR4 was sequenced in both directions. The sequence from the *Hin*dIII site at m.u. 7.0 to the *Sal*I site at m.u. 5.7 is presented in Fig. 2 (the sequence beyond the *Sal*I site was reported in Leisy *et al.* (1986) GenBank: m14885). Analysis of the sequence with the GRAIL suite of sequence analysis tools (Uberbacher, 1991) indicated the presence of several major open reading frames (Figs. 2 and 3a). (Where possible, ORFs are numbered the same as their homologs in the AcMNPV genome as reported by Ayres *et al.* (1994). When no homolog was present, the ORFs are named based on their size in nt.) ORFs with excellent coding potential were ORF 4 (146 amino acids, 16.2 kDa), ORF 5 (77 amino acids, 8.6 kDa), *lef-2* (204 amino acids, 22.7 kDa), ORF 393 nt (131 amino acids, 15.0 kDa), and ORF 189 nt (63 amino acids, 7.6 kDa). Predicted polypeptides encoded by ORFs entirely contained on pGR4 were used to search for ORFs from a colinear region of the AcMNPV genome (Ayres *et al.*, 1994) with the TFASTA program from the GCG software package (Devereux *et al.*, 1984). This search indicated the following amino acid sequence identities: ORF 4, 64.4% with AcMNPV ORF 4; ORF 5, 49.3% for 80 amino acids of AcMNPV ORF 5; LEF-2, 54.7% with AcMNPV LEF-2; homologs for ORF 393 nt and ORF 189 nt were not found in the AcMNPV genome.

Identification of the ORF essential for DNA replication located within the *Hin*dIII–*Xba*I fragment

In order to identify the ORF essential for DNA replication, several subclones of the 2.4-kb fragment were tested for their ability to transactivate replication of the reporter plasmid in the transient replication assay. The full-length pGR4 clone supported replication when cotransfected along with cosmid 9, pDNApol, pIE-1, and pHdN (data not shown), however, to much lower levels than when cotransfected with Op47B/E (Fig. 1c, lane 10). Similarly, subclone pCA30 was able to transactivate DNA replication to very low levels (Fig. 3b, lane 1; visible on longer exposures), indicating that neither ORF 4, ORF 393 nt, nor ORF 189nt (Fig. 3a) were essential for DNA replication. The ability of the subclone pCA35, which contains the entire *lef-2* gene and a partial ORF 5, to replace pGR4 (Fig. 3b, lane 2), indicated that ORF 5 was probably not required for DNA replication and implicated *lef-2* as the essential gene. DNA replication was stimulated in this experiment approximately 20-fold when pIE-2, a subclone of the pOp47B/E clone which contains the *ie-2*

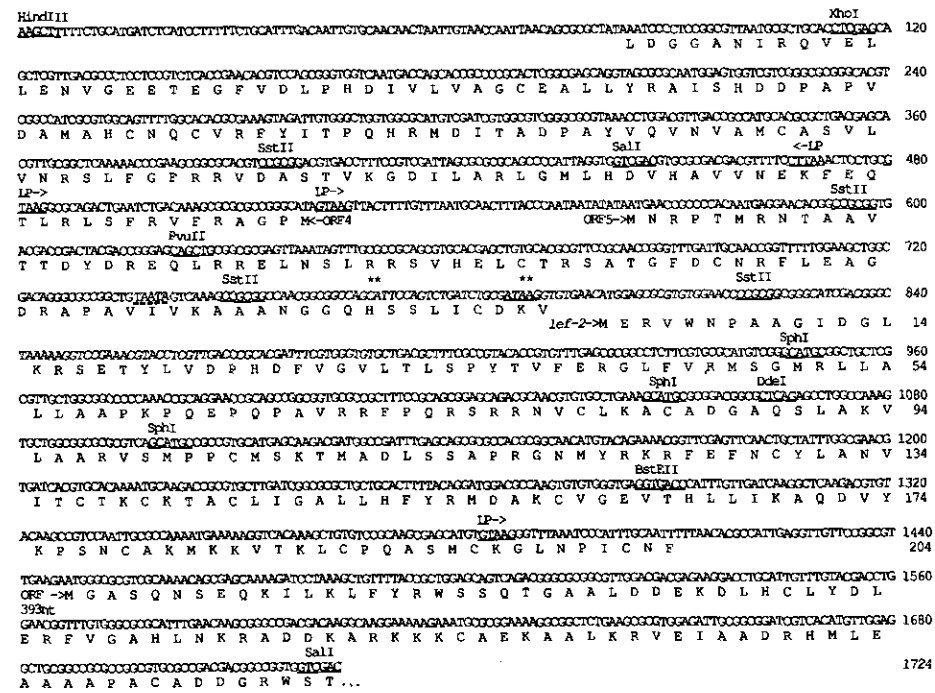

FIG. 2. Nucleotide sequence of the region from HindIII (m.u. 7.0) to SalI (m.u. 5.7) of the OpMNPV genome. The predicted amino acid sequences for major ORFs are shown below the nucleotide sequence. Numbering for the nucleotide sequence and the predicted amino acid sequence of lef-2 is shown to the right of each row. The transcriptional initiation sites of lef-2 transcripts are indicated by asterisks above the sequence. Selected restriction endonuclease sites and late promoter consensus sequences are underlined, the latter are also marked by arrows (→). A sequence (TAATA) implicated as an promoter for early lef-2 expression is underlined with a dashed line.

gene (Fig. 1b, m.u. 0–1.75) (Theilmann and Stewart, 1992a), was cotransfected along with a transfection mixture identical to that contained in lane 2 (Fig. 3b, lane 3). Finally, no DNA replication is observed when pCA35SphΔ, a clone with an internal deletion in the *lef-2* gene, was used along with the strong activator pIE-2 (Fig. 3b, lane 4). Therefore, *lef-2* is the essential replication gene contained within m.u. 5.2–7.0, and *ie-2* is a strong activator of OpMNPV DNA replication.

Northern blot analysis of *lef-2* expression

To examine the temporal regulation of *lef-2* transcription, Northern blot analyses were carried out. A deletion clone of pCA30, pCA39, was linearized with *Sst*II, and a labeled strand-specific probe complementary to an internal portion of the *lef-2* transcript was synthesized with T3 RNA polymerase (Fig. 4a). The labeled cRNA was hybridized to Northern blots of total RNA isolated from OpMNPV-infected Ld-652Y cells (m.o.i. of 10) at various times postinfection. Expression of a major *lef-2*-specific transcript of approximately 1.6 kb was first detected by 6 (visible on longer exposures, data not shown) to 12 hr postinfection (Fig. 4b). This RNA therefore is classified as an early transcript since it is expressed shortly before the onset of DNA replication which occurs at about 12–18 hr p.i. in OpMNPV (Bradford *et al.*, 1990). The steady-state levels of this transcript increased up to 48 hr p.i. and declined thereafter. This temporal pattern of regulation differs from those of two other essential OpMNPV DNA replication genes, *lef-1* (Ahrens and Rohrmann, 1995) and *lef-3* (Ahrens *et al.*, 1995a), both of which are expressed to higher levels early in the infection and only minimal transcript levels were detectable by 60 hr p.i.

Mapping the 5′ and 3′ ends of OpMNPV *lef-2* transcripts

Both the 5′ and 3′ termini of the *lef-2* transcript were mapped by S1 nuclease protection assays with RNA's isolated from infected cells at selected time points post-infection. Analysis of the nucleotide sequence upstream of the initiation codon of *lef-2* indicated the presence of a late promoter consensus motif (ATAAG near nt 795, Fig. 2) and a putative early transcriptional regulatory element resembling a TATA box (at about nt 740, Fig. 2). We therefore performed S1 nuclease protection assays on RNA from two time points where both early and late transcripts were expressed (18 and 48 hr p.i.). An 1150-bp 5′ end-labeled *Dde*I restriction enzyme fragment, covering sequences downstream of the ATG of *lef-2* and extending into sequences of the vector pKS (Fig. 4a), protected fragments 276 and 277 nt and 297 and 298 nt in length for both time points (Fig. 4c). The transcriptional initiation sites of the early *lef-2* transcripts therefore map to nucleotides 769 and 770, approximately 30 bp down-

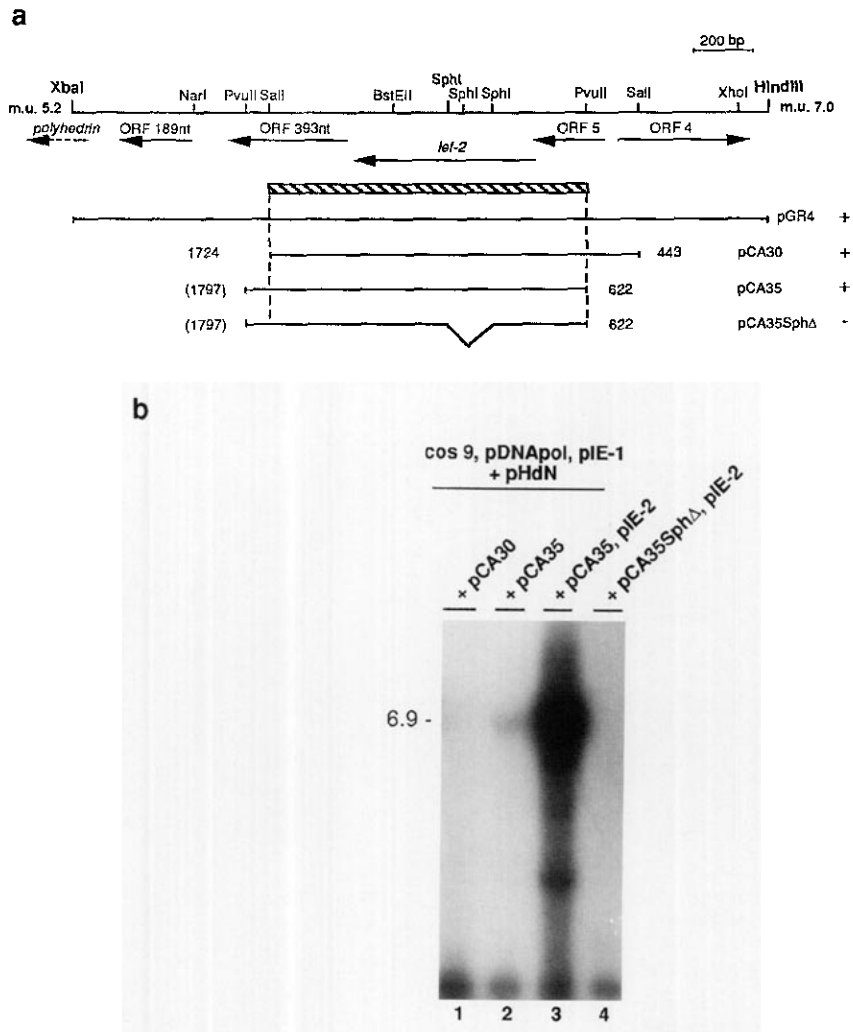

FIG. 3. Identification of the essential replication gene within m.u. 5.2–7.0 of the OpMNPV genome. (a) Map of the 2.4-kb pGR4 clone showing selected restriction endonuclease sites. The location and size of major open reading frames (ORFs) and a selection of subclones used to identify the essential ORF are shown below the map. The partially contained polyhedrin gene is represented by a dashed arrow. The ability of subclones to supply the essential replication gene is indicated by a + or − to the right of the diagram of each subclone. The vertical dashed lines and the crosshatched bar indicate the minimal region that supports replication. Numbers indicate the location of junctions of the subclones from the nucleotide sequence of this region (see Fig. 2). (b) Analysis of subclones of pGR4 for their ability to supply the essential replication gene. The replication signals of transfection mixtures containing cosmid 9, pDNApol, pIE-1, pHdN, and the following additional clones are shown in lanes 1–4: pCA30 (lane 1); pCA35 (lane 2); pCA35 and pIE-2 (lane 3); pCA35SphΔ and pIE-2 (lane 4). The size of linearized pHdN (in kb) is indicated to the left and right of the blot.

stream of a putative TATA box and 30 bp upstream of the initiation codon of *lef-2* (Fig. 2, asterisks indicate transcriptional initiation sites for the early and late *lef-2* transcripts). The late initiation sites map to nucleotides 790 and 791, which correspond to the nucleotides AA located within a late promoter consensus motif ATAAG (Fig. 2). Consistent with the transcription from different early and late regulatory elements, early transcript expression levels were higher than those of the late transcript at 18 hr, while by 48 hr expression of the late transcript reached higher levels (Fig. 4c).

The 3′ end of the *lef-2* transcript was mapped using two different 3′ end-labeled restriction enzyme fragments (Fig. 4a). RNA extracted 18 and 48 hr after infection protected a portion of the *Bst*EII–*Eco*RI restriction fragment of approximately 0.9 kb (data not shown). A polyadenylation signal is located approximately 770 bp downstream of the 3′ end of *lef-2* and 890 bp downstream of the *Bst*EII site (see sequence GenBank:m14885). In order to precisely map the 3′ end of *lef-2*, a 3′ end-labeled *Nar*I–*Eco*RI restriction fragment was used (Fig. 4a), which in addition to some nonspecific bands protected fragments 225–227 nts in size for both 18 and 48 hr time points (Fig. 4d). A fragment of similar length is protected using RNA from an additional time point where only the late transcript should be expressed (60 hr p.i.) (Fig. 4d). The

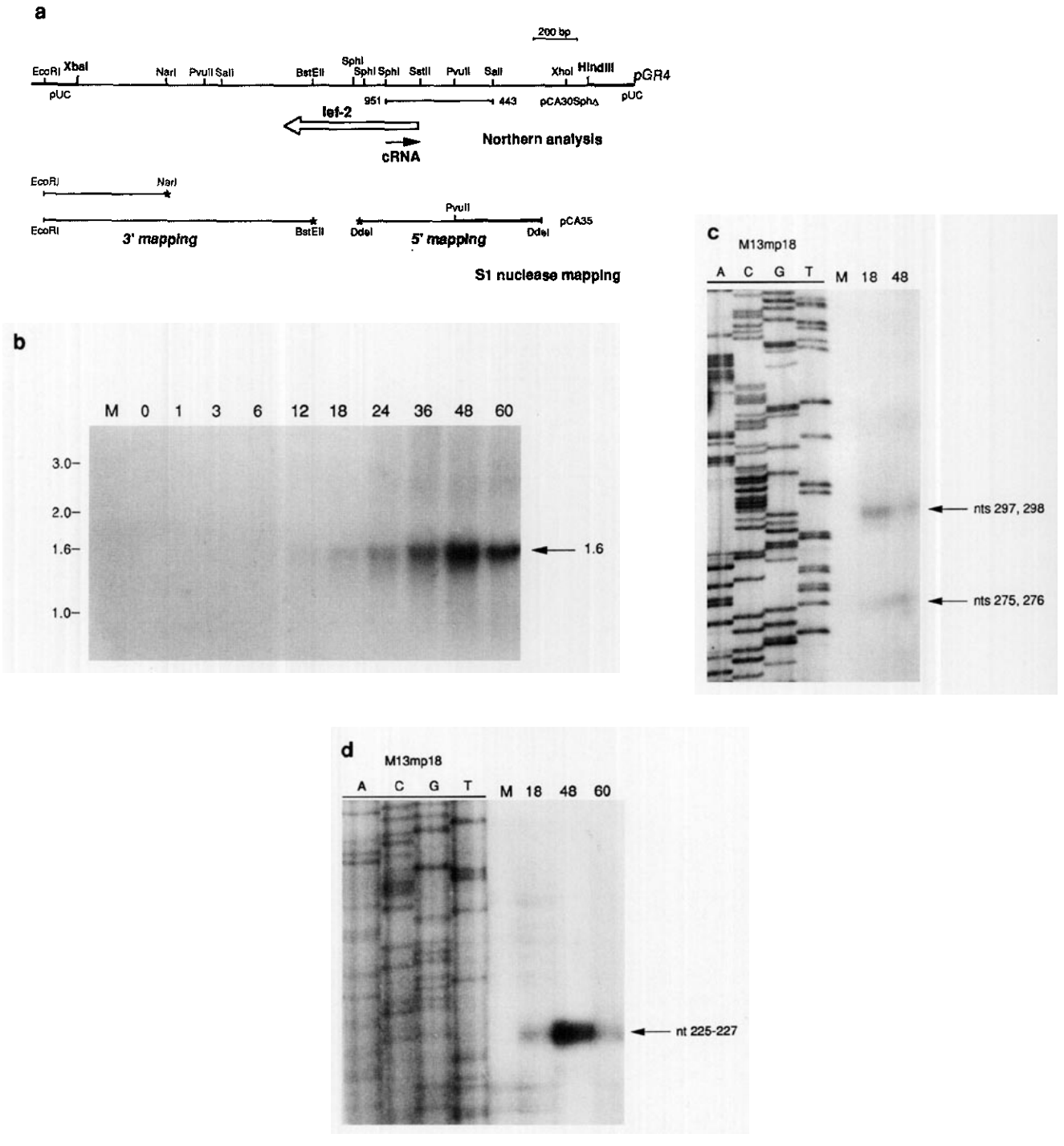

FIG. 4. Transcriptional mapping of OpMNPV *lef-2* transcripts. (a) Map of pGR4 with a schematic representation of *lef-2* (open arrow) and the plasmid pCA39 (numbers indicate the location of the deletion junction from the sequence shown in Fig. 2), used to generate a strand-specific cRNA transcript (plain arrow). DNA fragments used to map the 5′ and 3′ ends of the *lef-2* transcripts in S1 nuclease protection assays are shown and were generated from pCA35 (5′ mapping) or pGR4 (3′ mapping). Asterisks indicate the position of the label on the complementary strand of the DNA fragment. (b) Northern blot analysis. Total RNA was extracted from Ld-652Y cells either mock-infected (M) or infected with OpMNPV at a m.o.i. of 10, at the hours postinfection indicated above the lanes. The locations of marker DNA (kb) are shown to the left of the blot and the size (in kb) and location of the major band corresponding to the *lef-2* transcript is indicated by an arrow on the right. (c) S1 nuclease mapping of the 5′ end of the *lef-2* transcript. RNA from mock-infected cells (M) or RNA extracted 18 and 48 hr postinfection hybridized to a 5′ end-labeled *DdeI* fragment were employed. The sequence ladder used for sizing of the protected fragments was generated with bacteriophage M13mp18 DNA using the M13 (−40) sequencing primer. Major protected fragments are indicated by arrows and numbers that correspond to the size of the protected fragments. See Fig. 2 for location of the transcriptional initiation sites of *lef-2*. (d) S1 nuclease mapping of the 3′ end of the *lef-2* transcript. RNA from mock-infected cells (M) or RNA extracted 18, 48, and 60 hr postinfection (18, 48, 60) hybridized to a 3′ end-labeled *EcoRI/NarI* fragment is shown. Major protected fragments are indicated by the arrow (see text).

```
AcLEF-2   1-      MANASY NVWSPLIRAS CLDKKATYLI DPDDFIDKLI LTPYTVFYNG GVLVKISGLR - 56
OpLEF-2   1-         ME RVWNPAAGID GLKRSETYLV DPHDFVGVLT LSPYTVFER- GLFVRMSGMR - 51
LdLEF-2   1- MTSSSCPRAS LNYRPAMKAS DVDPDAEYAV PLEHF--DVE VSPYTVFERG GTCVRVSGRR - 58

AcLEF-2  57- LYMLL----- ---TAP-PTI NEIKNSNFKK RSKRNICMKE CVEGKKNVVD MLNNKINMPP -107
OpLEF-2  52- LLALL----- ---AAPKPQE PQPAVRRFPQ RSRRNVCLKA CADGAQSLAK VLAARVSMPP -103
LdLEF-2  59- LACLLRNGSR GESAPAPAAA AASAGQPGRK RSCKNVCFKG -ATSRRELER TLTARVNLPP -117

AcLEF-2 108- CIKKILNDLK ENNVPRGGMY RKRFILNCYI ANVVSCAKCE NRCLIKALTH FYNHDSKCVG -167
OpLEF-2 104- CMSKTMADL- -SSAPRGNMY RKRFEFNCYL ANVITCTKCK TACLIGALLH FYRMDAKCVG -161
LdLEF-2 118- CMTGLLRQFE IRN--RGDRY RKRFVFNCYL INTTTCTACD RRCFVNAAAV LYERDEKCVR -175

AcLEF-2 168- EVMHLLIKSQ DVYKPPNCQK MKTVDKLCPF AGNCKGLNPI CNY -210
OpLEF-2 162- EVTHLLIKAQ DVYKPSNCAK MKKVTKLCPQ ASMCKGLNPI CNF -204
LdLEF-2 176- EMMSLL-RRE DCYKPPNCSK MSQ-ESLCFK SGACRGTNPL CNF -216
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FIG. 5. Alignment of the LEF-2 sequences from OpMNPV, AcMNPV, and LdMNPV. The alignment was generated with the PILEUP program of the GCG package of sequence analysis software (Devereux *et al.*, 1984) (Version 7.2-UNIX, 1992). Identical amino acids are shaded.

3′ ends of both early and late *lef-2* transcripts thus map to 21–23 nt downstream of a polyadenylation signal (AATAAA), which is located approximately 355 nt downstream of the *Sal*I site at the end of the sequence shown in Fig. 2. With the exception of *ie-1*, no spliced baculovirus genes have been reported. Therefore, the data for the 5′ and 3′ mapping of *lef-2* predict early and late transcripts of approximately 1.45 kb (not including a poly(A) tail), which is in good agreement with the transcript size of 1.6 kb detected by Northern analysis.

Database search

All available databases were searched with the predicted amino acid sequence of OpMNPV *lef-2* (204 amino acids, 22.7 kDa) using the BLAST (Altschul, 1990) network service of the National Center for Biotechnology Information and the FASTA (Pearson and Lipman, 1988) program from the suite of sequence analysis software of the Genetics Computer Group in Wisconsin (Devereux *et al.*, 1984) (Version 7.2-UNIX, 1992). Significant homology to a number of baculovirus LEF-2 proteins was observed, and alignments performed with GCG's GAP program revealed the following amino acid identities to OpMNPV LEF-2: *Anticarsia gemmatalis* MNPV LEF-2 (Zanotto *et al.* 1992) (the reported sequence encodes the carboxyl-terminal 106 amino acids), 77.9%; AcMNPV LEF-2 (Ayres *et al.*, 1994) (210 amino acids, 23.2 kDa), 54.7%; LdMNPV LEF-2 (Bjornson and Rohrmann, 1992) (216 amino acids, 24.1 kDa) 44.4%. An alignment of the three full-length LEF-2 homologs generated with GCG's PILEUP program is shown in Fig. 5. No apparent motifs found in other replication proteins could be identified by computer analyses in the LEF-2 proteins.

Summary of essential OpMNPV DNA replication genes and identification of additional genes stimulatory for DNA replication

With the identification of *ie-1* and *lef-2*, we demonstrate that OpMNPV encodes a total of six genes essential for DNA replication. These include: *DNA polymerase, helicase, ie-1, lef-1, lef-2,* and *lef-3.* Transfection of all six replication genes (*lef-1* is supplied by pHdN) generates a weak replication signal (Fig. 6, lane 1). Cotransfection of *ie-2* along with the six essential genes highly stimulated DNA replication of both the reporter plasmid and the other plasmids (Fig. 6, lane 2). The transient DNA replication assay is therefore not origin-specific, al-

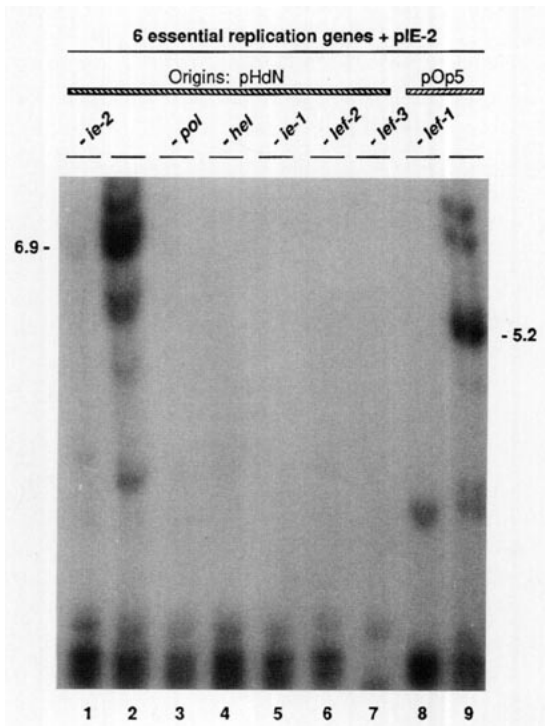

FIG. 6. Activation of replication by *ie-2* and summary of essential replication genes. The autoradiogram shows the results of replication assays with six essential genes and the viral activator *ie-2.* Plasmids containing two OpMNPV replication origins (pHdN and pOp5) (Ahrens *et al.*, 1995b) were used as reporter plasmids because pHdN contains *lef-1* in addition to an origin of replication. Replication signals of cotransfection mixtures that lack each essential replication gene are shown in lanes 3–9 (each omitted gene is indicated above the bar). The replication signal of a mixture lacking the *ie-2* gene is shown in lane 1 (*ie-2* is contained in all other lanes to increase the sensitivity of the assay). The sizes (in kb) of linearized pHdN and pOp5 are indicated at the left and the right of the blot, respectively.

though plasmids that contain putative replication origins replicate to higher levels. This is similar to what has been described for the AcMNPV transient replication assay (Kool *et al.*, 1994). Omission of each essential replication gene from this mixture of seven clones completely eliminated the replication signal (Fig. 6, lanes 3–8) (pOp5, used as the reporter plasmid in the experiments shown in lanes 8 and 9, contains a second OpMNPV origin of replication (Ahrens *et al.*, 1995b) and does not supply *lef-1*, which is contained on pHdN).

During these investigations, we also observed that other regions of the OpMNPV genome had the ability to stimulate DNA replication. Early on, we discovered that omission of cosmids 1 and 27 and of cosmids 1 and 13 from a cotransfection mixture containing all eight overlapping cosmid clones and pHdN resulted in reduced levels of replication of the reporter gene. These data suggested that a gene(s) stimulatory for DNA replication was contained within the region of the OpMNPV genome covered by cosmids 1 and 13 (Fig. 1a, *Hin*dIII-S to *Hin*dIII-K, m.u. 12.8–25.4). We subsequently determined that the *Hin*dIII-K region (Fig. 7a, m.u. 22.4–25.4) was responsible for this stimulation of DNA replication (Fig. 7b, lane 1). Subclones of *Hin*dIII-K were tested for their ability to replace *Hin*dIII-K in a transfection mixture containing the six essential OpMNPV replication genes. Neither pCA16, pCA17, nor pCA18 (Fig. 7a) were able to stimulate DNA replication (Fig. 7b, lanes 2–4). However, pOp-*iap*, a clone containing a functional analog of the AcMNPV *p35* gene, which inhibits apoptosis in AcMNPV-infected *S. frugiperda* cells, was able to replace *Hin*dIII K in stimulating DNA replication of pHdN (Fig. 7b, lane 5).

As mentioned above, the experiments shown in Fig. 3c had identified *ie-2* as a strong stimulator of DNA replication. *Ie-2* was initially tested because we had found that pOp47B/E had a strong stimulatory effect on DNA replication (Fig. 1b, lane 8 versus 9). However, in addition to *ie-2*, pOp47B/E also contains *p*34, (Theilmann and Stewart, 1992b) (Fig. 7a). OpMNPV *p*34 has been shown to transactivate the *ie-2* promoter (Wu *et al.*, 1993a). Furthermore, AcMNPV *p34* homolog, *PE-38*, was shown to have a modest stimulatory role in the AcMNPV replication system (Kool *et al.*, 1994). Therefore, we tested *ie-2* and *p*34 individually and together and quantified the relative replication levels of pHdN from several independent replication assays with a phosphorimager. These data revealed that *p*34 could stimulate DNA replication about 1.5- to 2-fold (Fig. 7c, lane 2 versus 1 (the exposure of this blot yielded only a very weak replication signal for the six essential genes)), while *ie-2* was a much stronger activator (lane 3) and caused an average 10- to 12-fold stimulation. Surprisingly, addition of *p*34 to a transfection mixture containing the six essential replication genes and *ie-2* results in a 3- to 4-fold inhibition of the strong stimulation by *ie-2* (Fig. 7c, compare lanes 4 and 3), an effect that is not observed in the transient AcMNPV DNA replication assay (Kool *et al.*, 1994). Furthermore, addition of the *Hin*dIII-K fragment, which contains the Op-*iap* gene, to a mixture of the six essential genes stimulated the replication levels of pHdN on average 3- to 4-fold in a number of independent experiments (Fig. 7c, lane 5). The six essential and three stimulatory genes for OpMNPV DNA replication are summarized in Table 1. On the basis of the homology to AcMNPV *iap1* and the existence of a second *iap* gene (Op-*iap2*) in the OpMNPV genome (Ahrens *et al.*, 1995a), we propose to term Op-*iap* (Birnbaum *et al.*, 1994) Op-*iap1*.

DISCUSSION

In this report, we have identified two genes, (*lef-2* and *ie-1*) that are essential for OpMNPV DNA replication. *Lef-2* is homologous to AcMNPV *lef-2*, a gene originally identified as being essential for late and very late transcription (Passarelli and Miller, 1993), and which subsequently was shown to be essential for AcMNPV DNA replication (Kool *et al.*, 1994; Lu and Miller, 1995). OpMNPV *lef-2*, the first of the baculovirus *lef-2* genes to be transcriptionally mapped, is expressed before the onset of DNA replication and an additional late transcript initiates from within a baculovirus late promoter motif (ATAAG). Late promoter motifs are also found upstream of the LdMNPV and AcMNPV *lef-2* initiation codons (LdMNPV, 289 nt upstream; AcMNPV, 60 (TTAAG) and 165 nt upstream). The conservation of these late promoter elements suggests that *lef-2* may have an important role in late events of the baculovirus replication cycle.

Ie-1 encodes a major viral transactivator, is expressed both very early (2 hr p.i.) and late in the infection, and has been shown to upregulate its own expression (Theilmann and Stewart, 1991). The steady state levels of its gene product increase up to 120 hr p.i. (Theilmann and Stewart, 1994). It is homologous to AcMNPV *ie-1*, whose gene product has been functionally characterized (Kovacs *et al.*, 1992). The N-terminal 145 amino acids of the polypeptide contain an acidic activation domain similar to those found in other transactivators like yeast GAL4 (Keegan *et al.*, 1986) and GCN4 (Hope and Struhl, 1986), as well as VP16 (Triezenberg *et al.*, 1988). The carboxyl-terminal portion contains a domain involved in DNA binding and prediction of the secondary structure of amino acids 485 to 582 suggested it may fold into a helix–loop–helix-like DNA-binding motif (Kovacs *et al.*, 1992). Extracts of cells transfected with AcMNPV IE-1 have been shown to bind to *hr* sequences that function as origins of replication (Guarino and Dong, 1991; Leisy *et al.*, 1995). *Ie-1* could be essential for DNA replication because of its role in stimulating expression of the other replication genes. However, it could also play a role as an origin binding protein and may serve to nucleate the formation of the replication initiation complexes. Our data demonstrate

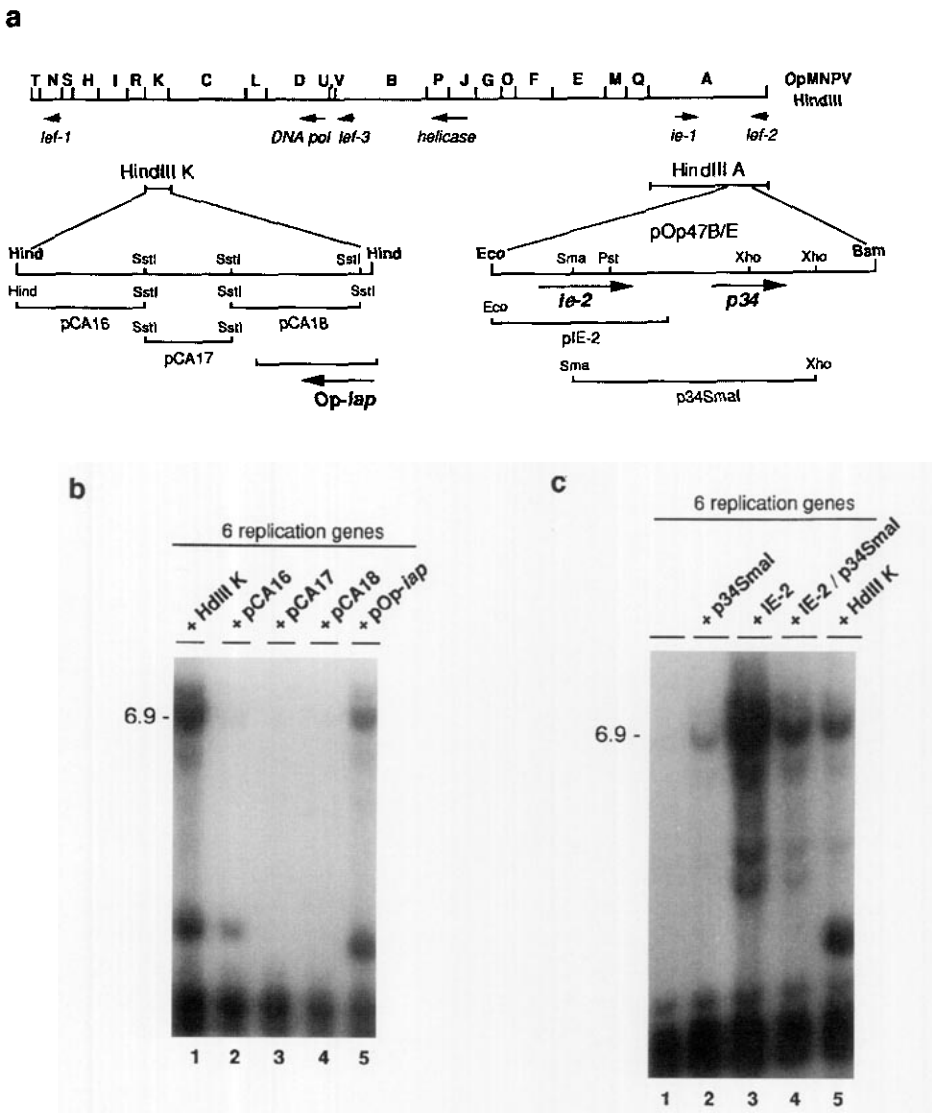

FIG. 7. Identification of additional genes that stimulate OpMNPV DNA replication. (a) *Hin*dIII map of the OpMNPV genome showing the location of the six essential replication genes. The *Hin*dIII fragments K and A which contain genes that activate DNA replication and a variety of subclones used to identify these stimulatory genes (represented by arrows) are shown at a larger scale. (b) Analysis of activation by subclones of the *Hin*dIII-K region. Each lane shows the results of transfection with plasmids containing the six essential replication genes (pDNApol, phel, pIE-1, pHdN, pCA35, pCA29) and an additional clone derived from the *Hin*dIII-K region, indicated above each lane. (c) Effects of stimulatory genes (*ie-2*, *p*34, and Op-*iap*) on DNA replication. Lanes show the results of cotransfecting the six essential genes along with subclones of pOp47B/E or *Hin*dIII-K as indicated above each lane. The size of linearized pHdN (in kb) is shown at the left of each blot in b and c.

that the gene product of the nonspliced *ie-1* transcript is essential for OpMNPV DNA replication. Experiments with a larger clone (pOp47HS-7.4, (Theilmann and Stewart, 1994)) that includes sequences contained on pIE-1 and additional upstream sequences to m.u. 90.5, resulted in a stimulation of the replication signal (data not shown). This clone contains the *ie-0* exon and may indicate that the spliced gene stimulates replication.

We have also identified three genes that stimulate DNA replication. Both *ie-2* and *p*34 have been reported to be transcriptional activators. In particular, OpMNPV *p*34 has been shown to transactivate the *ie-2* promoter approximately twofold (Wu *et al.*, 1993a). The AcMNPV PE-38 gene has been shown to stimulate expression of the AcMNPV *helicase* gene (Lu and Carstens, 1993). *p*34 contains a number of motifs found in several other eukaryotic *trans*-activators, including a putative zinc finger binding domain, a glutamine-rich domain, and a leucine zipper (Wu *et al.*, 1993a). The stimulation of replication is modest for both OpMNPV and AcMNPV (Kool *et al.*, 1994; Lu and Miller, 1995). In the OpMNPV replication system, the slight (about twofold) stimulation of replication by *p*34 may reflect the twofold upregulation of the *ie-2* promoter by *p*34 (Wu *et al.*, 1993a). Furthermore, in our hands *p*34 appeared to inhibit the strong stimulation caused by IE-2 by three- to fourfold.

In contrast to the AcMNPV replication system where

TABLE 1

OpMNPV Replication Genes

Gene	Map units	MW[a]	Amino acids	Function(s)	Motifs[b]	Essential (E) Stimulatory (S)
DNA pol	46.5–44.2	112.6	985		DNA Pol	E
helicase	64.9–62.1	140.5	1222		Helicase	E
ie-1	95.8–97.1	64.3	560	Transcriptional activator	SSB	E
lef-1	10.4–9.85	27.9	243			E
lef-2	6.65–6.15	22.7	204			E
lef-3	49.3–48.4	42.6	373	SSB[c]	SSB	E
ie-2	0.5–1.45	45.7	405	Transcriptional activator		S
p34	2.15–2.85	34.7	307	Transcriptional activator		S
Op-*iap1*	25.4–24.8	30.1	268	Inhibits apoptosis		S

[a] Molecular weights of replication proteins are predicted with GCG's PEPTIDESORT program.
[b] Listed motifs are confined to motifs commonly found in components of replication systems (Kornberg and Baker, 1992).
[c] Hang *et al.* (1995).

p35 shows the highest level of stimulation, *ie-2* is the strongest stimulator of OpMNPV DNA replication. IE-2 has been demonstrated to upregulate expression of the p8.9 and *ie-1* promoters and in addition has been shown to be autoregulatory (Theilmann and Stewart, 1992a; Wu *et al.*, 1993b). Its gene product can be detected from 2 hr p.i. until 36 hr p.i. on Western blots (Theilmann and Stewart, 1994). Like *p34*, IE-2 has both zinc finger and leucine zipper motifs (Theilmann and Stewart, 1992a) and may directly activate replication genes in addition to *ie-1* or may interact with cellular transcription factors via its leucine zipper motif and form a heterodimeric activator. As noted above, *p34* inhibits the high levels of activation of DNA replication by IE-2. This interference may even be more pronounced *in vivo,* since at late times during OpMNPV infection a truncated form (20 kDa) of the full-length *p34* (34.7 kDa) is expressed by transcriptional initiation from a late promoter located within the full-length *p34* ORF. This 20-kDa protein retains the leucine zipper motif but lacks both the basic region and the zinc finger DNA-binding domain, does not transactivate the *ie-2* promoter (Wu *et al.*, 1993a), and may inhibit stimulation of replication by forming a complex with and inactivating IE-2.

The third gene we identified that stimulates OpMNPV DNA replication is called Op-*iap.* Op-*iap* has been shown to substitute for the AcMNPV *p35* gene in blocking apoptosis induced by infection of *S. frugiperda* cells with an AcMNPV mutant lacking the apoptosis-inhibiting *p35* gene (Birnbaum *et al.*, 1994). Although Op-*iap* and *p35* can functionally replace each other, they do not share sequence homology. *P35* had a strong stimulatory effect in the AcMNPV replication system in one study (Kool *et al.*, 1994) and was essential for replication in another study (Lu and Miller, 1995). These observations suggest that expression of one or more of the AcMNPV replication genes causes apoptosis in transfected *S. frugiperda* cells. If the Op-*iap* gene blocks apoptosis in transfected *L. dispar* cells, it suggests that the transfection and subsequent expression of OpMNPV replication genes has only a limited capacity to induce apoptosis in this cell line.

Our analyses indicated that the OpMNPV genome contains six genes essential for DNA replication including *DNA polymerase, helicase, ie-1, lef-1, lef-2,* and *lef-3.* Homologs to each of these genes have been also been shown to be essential for AcMNPV DNA replication in a similar transient assay protocol (Kool *et al.*, 1994; Lu and Miller, 1995). However, the relative importance of the stimulatory genes differs in the two viral replication systems. We have demonstrated that three genes stimulate DNA replication, including *p34, ie-2,* and Op-*iap,* with *ie-2* having the most profound stimulatory effect. In contrast, *ie-2, PE-38, and p35* have been shown to be stimulatory for AcMNPV in transient DNA replication assays with *p35* having the most profound effect (Kool *et al.*, 1994). Another gene, *lef-7,* has also been reported to stimulate AcMNPV DNA replication (Lu and Miller, 1995). A homolog to this gene was not detected with our OpMNPV assay, but we cannot rule out the possibility that additional OpMNPV genes may be stimulatory for DNA replication.

The replication assay we employed is limited by the fact that it very likely does not result in the production of functional viral genomes. If the viral genome replicates in a manner similar to origin-containing plasmid DNA, with the production of long concatemers of the genome (Leisy and Rohrmann, 1993), then other genes required for the resolution of these structures are likely present which would not have been detected by our replication assay. Furthermore, the assay is not origin-specific and results in nonspecific plasmid replication. This suggests that a high degree of regulation is exerted when genes that are linked on a genome are expressed under natural infection conditions and that under these natural conditions a number of other genes could modulate the repli-

cation process. In addition, we cannot rule out the involvement of host factors in DNA replication.

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